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O^6-Methylguanine Is a Critical Determinant of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone Tumorigenesis in A/J Mouse Lung[1]

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ABSTRACT

The relative importance of the two α-hydroxylation pathways in the tumorigenicity of the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), was examined in the A/J mouse lung. Methyl hydroxylation, which results in DNA pyridyloxobutylation, was investigated with 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc) and N'-nitrosonornicotine. Methylene hydroxylation, which leads to DNA methylation, was studied by using acetoxymethylmethylnitrosamine (AMMN). The tumorigenic activities of these compounds were compared to that of 10 μmol NNK at doses that yielded similar or greater adduct levels 24 h after exposure. The methylating agent AMMN was more tumorigenic than the pyridyloxobutylating agents, NNKOAc and N'-nitrosonornicotine. NNKOAc enhanced the tumorigenic activity of AMMN when the two compounds were given in combination. These results suggested that DNA methylation was more important than DNA pyridyloxobutylation in A/J mouse lung tumor induction by NNK and that pyridyloxobutylation enhanced the activity of the methylation pathway. However, the tumorigenicity of 10 μmol NNK could not be reproduced by AMMN ± NNKOAc at doses that yielded similar levels of DNA adducts 24 h after exposure. Therefore, a second study was conducted in which the persistence of O^6-methylguanine in lung DNA following various doses of NNK or AMMN ± NNKOAc was compared to the tumorigenicity of these treatments. A strong correlation was observed between lung tumor yield and levels of O^6-methylguanine at 96 h for NNK and AMMN ± NNKOAc ($r = 0.98$). The ability of NNKOAc to increase the tumorigenic activity of AMMN was attributed to its ability to enhance the persistence of O^6-methylguanine in lung DNA. These results demonstrate that the formation and persistence of O^6-methylguanine are critical events in the initiation of A/J mouse lung tumors by NNK. They also suggest that DNA pyridyloxobutylation by NNK can increase the persistence of this promutagenic base in lung DNA.

INTRODUCTION

The tobacco-specific nitrosamine, NNK,[3] is present in significant quantities in tobacco products (1). This compound is a powerful pulmonary carcinogen in laboratory animals (2–4) and is believed to play a role in tobacco-related cancers in humans. NNK is a stable compound which requires metabolic activation in order to elicit its tumorigenic effects. It has two α-hydroxylation pathways through which it can be activated to DNA reactive species (Fig. 1). Methylene hydroxylation generates 4-hydroxy-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone which decomposes to methanediazohydroxide. This pathway leads to DNA methylation (5). Methyl hydroxylation yields 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone which decomposes to 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide. This compound pyridyloxobutylates DNA (6).

Methyl DNA adducts derived from NNK include 7- and O^6-methylguanine (5, 7). O^6-Methylguanine can induce G-C to A-T transitions (8). A strong correlation has been observed between lung tumor formation and levels of this adduct in Clara cells of NNK-treated rats (9). On the other hand, little is known about the biological activity of adducts resulting from DNA pyridyloxobutylation. A major portion of these adducts are chemically unstable and decompose to HPB under acid or enzyme DNA hydrolysis conditions. Despite their instability in DNA hydrolysates, the adducts persist in intact DNA for at least 4 weeks both *in vivo* and *in vitro*.[4] Methyl deuterium substitution markedly inhibited the mutagenic activity of NNK (10). In addition, compounds capable of DNA pyridyloxobutylation, such as 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone, are potent mutagens in *Salmonella typhimurium* (10). Therefore there is a potential role for pyridyloxobutyl adducts as well as methyl adducts in the carcinogenic activity of NNK.

We were interested in investigating the role of these two pathways in NNK-induced pulmonary tumorigenicity. This was studied by separating the pathways using model compounds that only methylate or pyridyloxobutylate DNA. The methylation route was studied using AMMN. This compound, in the presence of esterase, will generate the same methylating species formed upon methylene hydroxylation of NNK (Fig. 1) (11). NNKOAc was used to investigate the pyridyloxobutylation route. NNKOAc, following esterase-catalyzed hydrolysis, generates the diazohydroxide that leads to DNA pyridyloxobutylation (Fig. 1) (12). The availability of these model compounds permitted us to study each activation pathway individually.

We chose to investigate this question in the A/J mouse lung. We have previously demonstrated that a single i.p. dose of NNK induces a significant number of lung adenomas in A/J mice 16 weeks after exposure (4). The A/J mouse is a simple model that allows quantification of tumors in relatively short time periods. Furthermore, the single injection protocol eliminates complications of multiple dosing, thereby allowing us to study the biological consequences of the two activation pathways.

In order to assess the relative importance of each pathway in NNK-induced tumorigenicity, our goal was to determine tumor incidence in mice treated with doses of AMMN or NNKOAc that gave levels of methyl or pyridyloxobutyl DNA adducts approximating those produced by NNK. This goal was achieved by first measuring the time course of lung DNA methylation and pyridyloxobutylation following a single 10-μmol NNK dose. Doses of the model compounds that yielded levels of DNA methylation or pyridyloxobutylation similar to those produced by NNK were determined. Then, a comparative bioassay was conducted using doses of AMMN, NNKOAc, and NNK based on these data.

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[3] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNKOAc, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N'-nitrosonornicotine; AMMN, acetoxymethylmethylnitrosamine; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; MPB, 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide; HPLC, high performance liquid chromatography.

[4] L. A. Peterson, R. Mathew, S. E. Murphy, N. Trushin, and S. S. Hecht, Cancer Lett., in press, 1991.

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MATERIALS AND METHODS

Chemicals. NNK, NNN, NNKOAc, [5-^{3}H]NNKOAc, HPB, AcPB, and O^6-methylguanine were prepared as described (12–15). AMMN was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository. [5-^{3}H]NNK (1.2 Ci/mmol) and [5-^{3}H] NNN (1.1 Ci/mmol) were obtained from ChemSyn (Lexena, KS). 7-Methylguanine and guanine were purchased from Sigma Chemical Co. (St. Louis, MO).

Bioassays for Tumorigenicity. Female A/J mice, age 5 weeks, were purchased from The Jackson Laboratory (Bar Harbor, ME). They were housed in groups of 10 under standard conditions (4) and maintained on AIN-76A diet (Dyets, Inc., Bethlehem, PA; No. 100,000 with 5% corn oil). After 2 weeks, groups of 20 mice each were given i.p. injections of the appropriate compounds in saline. Two separate bioassays were conducted; Table 2 indicates the doses and compounds used in the initial bioassay whereas Tables 4, 6, and 7 denote treatments in the second bioassay. After 16 weeks, they were sacrificed by cervical dislocation and lung adenomas were counted.

Short Term Animal Studies. Female A/J mice were maintained on AIN-76A diet. At 7 weeks of age, the animals were given the appropriate compound in saline (i.p.). They were maintained on AIN-76A diet until they were sacrificed at the specified time by cervical dislocation. Lungs were removed, frozen immediately on dry ice, and stored at −80°C. Lung DNA was isolated using a modification of the Marmur method (6).

Time Course of DNA Pyridyloxobutylation by NNK. Five groups of four mice were treated with 10 μmol of [5-^{3}H]NNK (275.5 or 293.5 mCi/mmol) and were killed at 12, 24, 48, 96, and 144 h following the injection. All lungs of each group were pooled.

Dose Response for DNA Pyridyloxobutylation by [5-^{3}H]NNKOAc. Four groups of 4 mice were treated with [5-^{3}H]NNKOAc in saline (0.2 ml) and sacrificed 24 h later. All 4 lungs from each group were pooled. The doses each group received were: Group 1, 6.1 μmol (0.55 mCi); Group 2, 3.0 μmol (0.27 mCi); Group 3, 1.5 μmol (0.13 mCi); and Group 4, 0.6 μmol (0.05 mCi). A fifth group was treated with saline.

Time Course of DNA Pyridyloxobutylation by [5-^{3}H]NNKOAc. Three groups of 3 mice were treated with 5.5 μmol [5-^{3}H]NNKOAc (0.5 mCi) in saline (0.2 ml) and sacrificed 4, 8, or 12 h following injection. All 3 lungs from each group were pooled at each time point.

DNA Pyridyloxobutylation by [5-^{3}H]NNN. Eight mice were treated with 11 μmol [5-^{3}H]NNN (2.7 mCi) and sacrificed 24 h following injection. Lungs from 4 animals were combined for DNA isolation.

Time Course of DNA Methylation by NNK. Groups of 9–15 mice were treated with 10 μmol NNK and sacrificed at 1, 4, 8, 12, 24, 48, 96, 192, and 360 h following exposure. Lungs from 3–5 mice were pooled at each time point. There were 3 samples/time point.

Dose Response for DNA Methylation by AMMN. Five groups of 10 mice were given a single dose of AMMN in saline (0.2 ml). The doses were 0.5, 1.25, 2.5, 5, or 10 μmol AMMN. Lungs from 3 mice in each group were pooled.

DNA Methylation by NNK or AMMN ± NNKOAc. Sixteen groups of 27 mice were given single i.p. injections of saline solutions containing NNK or AMMN ± NNKOAc (0.15 ml) and divided into 3 subgroups for sacrifice at 4, 96, or 336 h after injection. Lungs from 3 mice were pooled at each time point. The doses and compounds given each group were: Group 1, 1 μmol NNK; Group 2, 1.5 μmol NNK; Group 3, 2 μmol NNK; Group 4, 2.5 μmol NNK; Group 5, 3 μmol NNK; Group 6, 5 μmol NNK; Group 7, 10 μmol NNK; Group 8, 0.5 μmol AMMN; Group 9, 1 μmol AMMN; Group 10, 1.5 μmol AMMN; Group 11, 2 μmol AMMN; Group 12, 0.5 μmol AMMN + 4.2 μmol NNKOAc; Group 13, 1 μmol AMMN + 4.2 μmol NNKOAc; Group 14, 1.5 μmol AMMN + 4.2 μmol NNKOAc; Group 15, 0.5 μmol AMMN + 4.2 μmol HPB; Group 16, 0.5 μmol AMMN + 4.2 μmol AcPB. In addition, there were 4 groups of 3 animals that were treated with 4.2 μmol NNKOAc, AcPB, HPB, or saline. These animals were sacrificed after 24 h and 3 lungs were pooled for each analysis.

Analysis of DNA for Levels of Pyridyloxobutylation and Methylation. Levels of DNA pyridyloxobutylation were determined as described (16). DNA was hydrolyzed in 0.1 M HCl at 70°C for 30 min (neutral thermal hydrolysis) or 6 h, 80°C). Levels of [5-^{3}H]HPB were analyzed by reverse-phase HPLC analysis with flowthrough detection of radioactivity (Flo-one/Beta radioflow detector; Radiomatic Instruments, Tampa, FL). Standards were added to neutralized hydrolysates and they were analyzed on a C_{18} reverse phase column (Whatman Partisil 5-ODS-3 cartridge column, 4.6 × 12.5 cm) eluted with solvents A (20 mM sodium phosphate buffer, pH 7) and B (95% methanol-5% H_2O) using a linear gradient from 100% A to 65% A over 60 min (flow rate, 1 ml/min). Levels of HPB released from DNA were determined from the radioactivity that co-eluted with standard. Guanine concentrations in the hydrolysates were determined by HPLC (5) and adduct levels were expressed as pmol HPB released/μmol guanine.

Neutral thermal and mild acid hydrolysates of DNA were prepared as previously reported (5). Levels of 7-methylguanine, O^6-methylguanine, and guanine were determined by HPLC analysis with fluorescence detection (LC 240 fluorescence detector; Perkin Elmer, Norwalk, CT). The hydrolysates were separated using two Partisil 10 SCX columns in tandem (Whatman, Inc., Clifton, NJ). Neutral thermal hydrolysates were eluted with 100 mM ammonium phosphate buffer, pH 2, and mild acid hydrolysates were eluted with the same buffer plus 10% methanol (flow, 1 ml/min). Quantitation was achieved using standard curves prepared for each analysis.

RESULTS

The relative importance of the methylene and methyl hydroxylation pathways of NNK metabolism in NNK-induced lung tumorigenesis was investigated using the model compounds AMMN and NNKOAc. Another tobacco-specific nitrosamine, NNN, was also investigated as a model for the pyridyloxobutylation route since hydroxylation at its 2′-position generates the same pyridyloxobutylating diazohydroxide as NNK and NNKOAc (6) (Fig. 1). Previous studies established that a single dose of 10 μmol NNK generated approximately 8–10 lung tumors/A/J mouse after 16 weeks (4). This dose was used as a standard in the present study. The tumorigenicities of the model compounds were compared at various doses including those that gave similar pulmonary DNA adduct levels as 10 μmol NNK.

DNA Pyridyloxobutylation by NNK, NNKOAc, and NNN. Levels of DNA pyridyloxobutylation by 10 μmol NNK were measured to establish doses of the pyridyloxobutylating m[illegible] compounds required for the comparative bioassay. The time course of DNA pyridyloxobutylation by NNK was determined by treating groups of mice with 10 μmol [5-^{3}H]NNK and sacrificing at various time points following exposure. The radiograms of the strong acid DNA hydrolysates contained several radioactive peaks in addition to [5-^{3}H]HPB as previously reported (14). These peaks result from metabolic pathways other than methyl hydroxylation of NNK (14). For the purposes of this study, we concentrated on the HPB-releasing adducts only.

The amounts of HPB released from DNA at various time points are shown in Fig. 2. In the mice treated with NNK, adduct levels peaked at 24 h; there were detectable levels 6 days after exposure. On the basis of these data, we used a 24-h time point to determine the doses of NNN and NNKOAc that produced similar levels of HPB-releasing adducts.

The amounts of HPB released from lung DNA of mice treated with 11 μmol [5-^{3}H]NNN or 10 μmol [5-^{3}H]NNK were similar (5.6 and 8.4 pmol [5-^{3}H]HPB released/μmol guanine, respectively). The dose response for adduct formation in lungs of [5-^{3}H]NNKOAc-treated mice has been published previously (14). Based on these data, lung DNA from mice treated with 4.2 μmol of NNKOAc or 10 μmol of NNK have approximately

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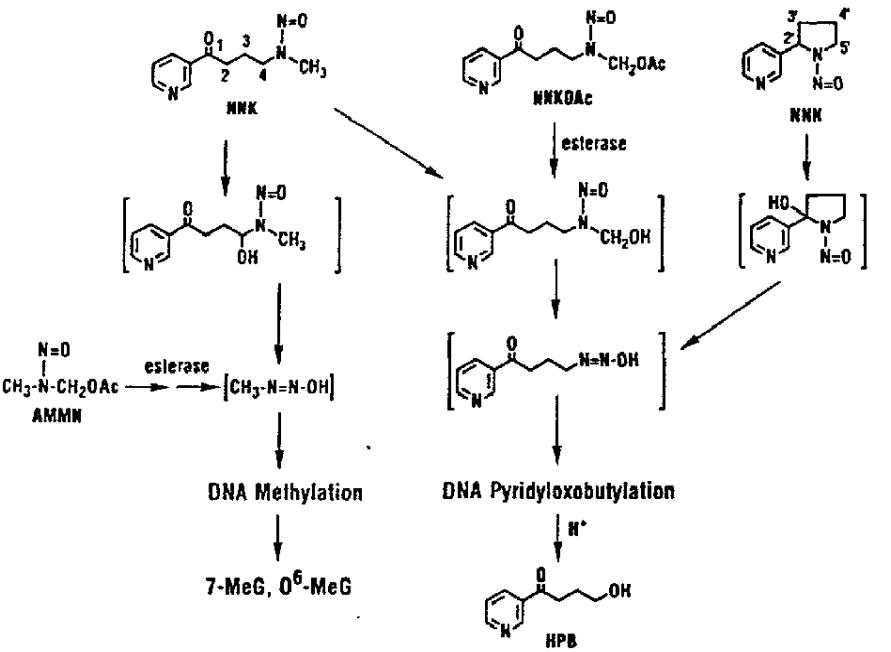

Fig. 1. Proposed bioactivation pathways of NNK to methylating and pyridyloxobutylating intermediates. 7-MeG, 7-methyl guanine; *O⁶-MeG*, *O⁶*-methylguanine.

the same amount of HPB-releasing adducts 24 h after treatment.

The time course of lung DNA adduct formation following injection of 5.5 μmol [5-^{3}H]NNKOAc is presented in Fig. 2. Due to limited amounts of radioactive compound, adduct levels were determined only at 4, 8, and 12 h. Unlike NNK, the amount of HPB-releasing adducts peaked 8 h after exposure. The area under the curve for HPB releasing adducts during the first 24 h for 5.5 μmol NNKOAc was roughly 3.7 times greater than that observed with 10 μmol NNK (Fig. 2). Therefore, 1.5 μmol NNKOAc (5.5 μmol ÷ 3.7) should give approximately the same overall amount of HPB-releasing adducts in lung DNA as observed with 10 μmol NNK during the first 24 h after injection. Based on these data, the following doses of pyridyloxobutylating agents were chosen for the comparative bioassay in A/J mice: 1.5, 2, 4.2, and 10 μmol NNKOAc; and 10 and 20 μmol NNN.

DNA Methylation by NNK and AMMN. The time course of DNA methylation by 10 μmol NNK is illustrated in Fig. 3. *O⁶*-Methylguanine persisted in pulmonary DNA up to 15 days. The highest levels of 7- and *O⁶*-methylguanine were observed 4 h after exposure. This contrasts with DNA pyridyloxobutylation which peaked 24 h after exposure. These differences suggest that there might be at least two different isozymes for NNK activation; one catalyzes methylene hydroxylation and the other catalyzes methyl hydroxylation. This is supported by *in vitro* microsomal studies that indicate there are different isozymes responsible for the two α-hydroxylation routes (16). Alternatively, these differences could reflect differences in initial rates of repair for these adducts.

In order to determine the dose of AMMN that yields similar levels of lung DNA methylation as 10 μmol NNK, a dose-response study was conducted, using a 24-h time point (Table 1). AMMN (0.5 μmol) yielded similar levels of *O⁶*- and 7-methylguanine as observed with 10 μmol NNK. This dose was used to determine the time course of DNA methylation by AMMN. While levels of *O⁶*-methylguanine were similar to those seen with 10 μmol NNK at 24 h, they fell to one-fifth the level produced by NNK in 2 weeks (Fig. 4). The area under the curve for *O⁶*-methylguanine levels up to 2 weeks for 10 μmol NNK was roughly 4 times greater than that observed for 0.5 μmol AMMN for the same time period. Therefore, the doses chosen for the comparative bioassay were 0.5 and 2 μmol AMMN. These doses should yield comparable levels of DNA methylation to those observed with 10 μmol NNK. A lower (0.1 μmol) and a higher dose of AMMN (5 μmol) were also included.

Initial Comparative Bioassay. The relative importance of the DNA pyridyloxobutylation and methylation routes was initially investigated by comparing the lung tumorigenicity of NNK to those of NNKOAc, NNN, and AMMN at various doses that generated similar levels of lung DNA alkylation as produced by 10 μmol NNK. We also included groups of mice that were treated with solutions containing both NNKOAc and AMMN. The results of this bioassay are summarized in Table 2.

The pyridyloxobutylating agents were weak lung tumorigens. NNN was only marginally active, if at all, at the doses tested.

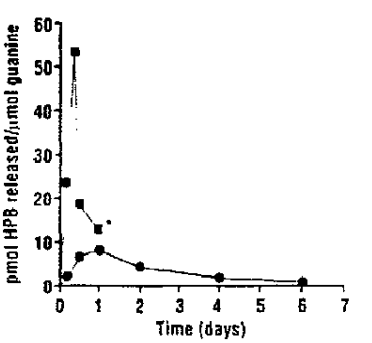

Fig. 2. Levels of [5-^{3}H]HPB released upon acid hydrolysis of lung DNA at intervals after injection of mice with 10 μmol [5-^{3}H]NNK (●) or 5.5 μmol [5-^{3}H]NNKOAc (■). *, estimation from the previously published dose-response study (14).

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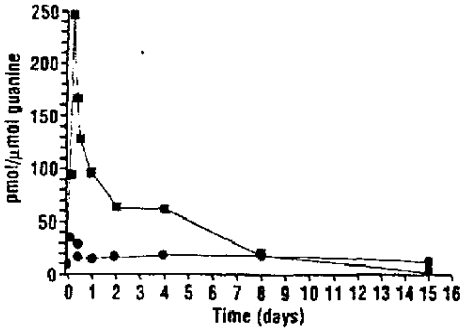

Fig. 3. Amount of 7-methylguanine (■) and O^6-methylguanine (●) at intervals after injection of mice with 10 μmol NNK.

Table 1 *Dose response for lung DNA methylation 24 h after treatment with AMMN*[a]

Compound	Dose (μmol)	pmol/μmol guanine	
		7-Methylguanine	O^6-Methylguanine
AMMN[b]	0.5	93.8	15.5
	1.25	207	35.7
	2.5	489	86.5
	5	632	130
	10	2640	514
NNK[c]	10	96.1 ± 26.3	14.3 ± 1.9

[a] Groups of A/J mice were treated with the indicated doses of AMMN or NNK in saline (i.p.) and sacrificed after 24 h. Five lungs were pooled for DNA isolation. See "Materials and Methods" for details.
[b] Mean of two values.
[c] Mean of three samples ± SD.

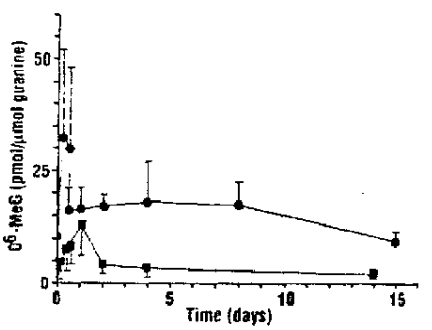

Fig. 4. Levels of O^6-methylguanine (*O^6-MeG*) in lung DNA at intervals after injection of mice with 10 μmol NNK (●) or 0.5 μmol AMMN (■). *Bars*, SD.

NNKOAc induced a dose-dependent increase in tumor formation. However, its activity was significantly less than that of 10 μmol NNK, even at doses that gave levels of DNA pyridyloxobutylation comparable or greater than those produced by NNK (1.5–4.2 μmol NNKOAc).

AMMN was more active in generating lung tumors than NNKOAc. There was a sharp increase in activity between 0.5 and 2 μmol. The tumor yield decreased at 5 μmol: this dose was toxic. The 0.5-μmol dose of AMMN was significantly less active than 10 μmol NNK despite similar levels of DNA methylation at 24 h.

Some combinations of AMMN and NNKOAc produced more tumors than either compound alone. This enhancement was observed only when 0.5 μmol AMMN was used. The increase was not dependent on NNKOAc concentration. The [illegible] NNKOAc and AMMN at doses that gave adduct levels similar to those of NNK at 24 h (4.2 and 0.5 μmol, respectively) did not reproduce the tumorigenic activity of 10 μmol NNK.

Comparison of O^6-Methylguanine Levels to Tumorigenicity. The comparative bioassay suggested that DNA methylation was more important than DNA pyridyloxobutylation in A/J mouse lung tumor induction. However, it was difficult to relate the tumorigenicity of AMMN to that of NNK in terms of DNA adduct levels. The 0.5-μmol dose of AMMN was less tumorigenic than 10 μmol NNK despite similar DNA adduct levels at 24 h. AMMN at 24 μmol, predicted from area under the curve calculations to yield similar overall levels of DNA methylation as 10 μmol NNK, was substantially more tumorigenic than 10 μmol NNK. The dose responses for adduct formation as well as lung tumor yield between these two doses of AMMN were not known. The substantial difference at time points greater than 24 h in O^6-methylguanine levels induced by 0.5 μmol AMMN and 10 μmol NNK suggested that persistence of this promutagenic base was important for lung tumorigenicity but our existing data were limited.

In addition, we were interested in determining how NNKOAc enhanced the tumorigenicity of AMMN. This increased activity suggested that DNA pyridyloxobutylation by NNK could modify the activity of DNA methylation by NNK. One possible mechanism through which NNKOAc could elevate the tumorigenic activity of AMMN is by increasing the persistence of O^6-methylguanine.

These questions were addressed by measuring levels of O^6-methylguanine in mouse lung 4, 96, and 336 h (14 days) following exposure to various doses of NNK as well as various doses of AMMN in the presence or absence of 4.2 μmol

Table 2 *Initial comparative bioassay of NNKOAc, NNN, AMMN, and NNK in A/J mice*[a]

Compound (μmol)	% of mice with lung tumors	Lung tumors/mouse
Saline	5	0.05 ± 0.05[b, e]
NNKOAc (1.5)	15	0.15 ± 0.1[b, e]
NNKOAc (2.0)	40[d]	0.4 ± 0.1[b, c, e]
NNKOAc (4.2)	50[f]	0.75 ± 0.2[b, c, f]
NNKOAc (10)	67[f]	1.1 ± 0.2[b, c, e, f]
NNN (10)	30[d]	0.3 ± 0.1[b, c, h]
NNN (20)	25	0.35 ± 0.2[b, e]
AMMN (0.1)	20	0.25 ± 0.1[b, e]
AMMN (0.5)	80[f]	1.3 ± 0.3[b, c, e]
AMMN (2.0)	100[f]	26.4 ± 1.5[b, c, e]
AMMN (5.0)	100[f]	4.9 ± 0.6[b, c, i]
AMMN (0.1) + NNKOAc (2.0)	30[d]	0.3 ± 0.1[b, c, h]
AMMMN (0.5) + NNKOAc (1.5)	85[f]	2.6 ± 0.5[b, c, f]
AMMN (0.5) + NNKOAc (2.0)	95[f]	3.2 ± 0.4[b, c, f]
AMMN (0.5) + NNKOAc (4.2)	100[f]	3.1 ± 0.4[b, c, f]
AMMN (2.0) + NNKOAc (2.0)	90[f]	25.7 ± 2.5[b, c, e]
NNK (5.0)	90[f]	3.1 ± 0.5[b, e]
NNK (10)	100[f]	8.2 ± 0.9[c, e]

[a] Groups of 20 mice (average starting weight, 20.1 ± 1.6 g) were given a single i.p. injection of the appropriate compound in saline at the indicated dose. After 16 weeks, the animals were sacrificed and lung adenomas were counted. The tumor counts are expressed as the mean ± SE.
[b] Statistically different from 10 μmol NNK, Student's *t* test, $P < 0.01$.
[c] Statistically different from 5 μmol NNK, Student's *t* test, $P < 0.05$.
[d] Statistically different from control, χ^2 test, $P < 0.05$.
[e] Statistically different from control, $P < 0.01$.
[f] Statistically different from control, χ^2 test, $P < 0.01$.
[g] Based on 30 animals.
[h] Statistically different from control, Student's *t* test, $P < 0.05$.
[i] Based on 11 animals.
[j] Statistically different from 0.5 μmol AMMN, Student's *t* test, $P < 0.01$.

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Table 3 *Levels of O⁶-methylguanine in mouse lung DNA 4, 96, and 336 h following treatment with NNK*

NNK[a] (μmol)	pmol O⁶-methylguanine/μmol guanine[b]		
	4 h	96 h	336 h
1	3.3 ± 0.7 (3)	1.6[c, d] (2)	<1.3 (4)[e]
1.5	6.8 ± 0.1 (3)	3.4 ± 0.4 (3)	<1.7 (4)
2	6.3 ± 0.6 (3)	2.9[c] (2)	<1.5 (4)
2.5	7.2 ± 1.2 (3)	4.4 ± 0.5 (3)	2.2 ± 0.2 (3)
3	6.6 ± 0.7 (3)	5.0 ± 2.0 (3)	2.0 ± 0.6 (3)
5	12.5 ± 0.4 (3)	10.2 ± 1.3 (3)	4.2 ± 0.4 (3)
10	21.3 ± 3.8 (3)	20.5 ± 5.1 (3)	9.4 ± 0.7 (4)

[a] Groups of 27 mice were given a single i.p. dose of NNK in saline (0.15 ml). Each group was divided into 3 subgroups for sacrifice at 4, 96, or 336 h postinjection. The lungs were pooled into 3 groups/time point for DNA isolation. See "Materials and Methods" for details.
[b] Mean ± SD. Numbers in parentheses, number of samples analyzed.
[c] Mean of 2 samples.
[d] A third sample did not have detectable levels of O⁶-methylguanine.
[e] Detection limits were 0.45 pmol O⁶-methylguanine. The amount of DNA analyzed ranged from 0.2 to 0.5 mg.

Table 4 *Dose response for lung tumor induction by NNK[a]*

NNK (μmol)	% of mice with lung tumors	Lung tumors/mouse	Tumors/O⁶-methylguanine at 96 h[b]
0	10	0.1 ± 0.1	
1	21	0.2 ± 0.1[c]	0.20
1.5	40[d]	0.5 ± 0.1[e]	0.13
2	30	0.3 ± 0.1	0.10
2.5	55[f]	0.9 ± 0.3[g]	0.20
3	90[f]	1.9 ± 0.4[g]	0.37
5	95[f]	3.4 ± 0.4[g]	0.34
10	95[f]	6.3 ± 0.6[g]	0.31

[a] Groups of 20 mice (average starting weight 20.1 ± 1.6 g) were treated with the appropriate compound at the indicated dose (in saline, i.p.). After 16 weeks, the animals were sacrificed and lung tumors were counted. The tumor counts were expressed as the mean ± SE.
[b] The ratio was obtained using O⁶-methylguanine levels presented in Table 3.
[c] Based on 19 animals.
[d] Statistically different than control, χ^2 test, $P < 0.05$.
[e] Statistically different than control, Student's t test, $P < 0.05$.
[f] Statistically different than control, χ^2 test, $P < 0.01$.
[g] Statistically different than control, Student's t test, $P < 0.01$.

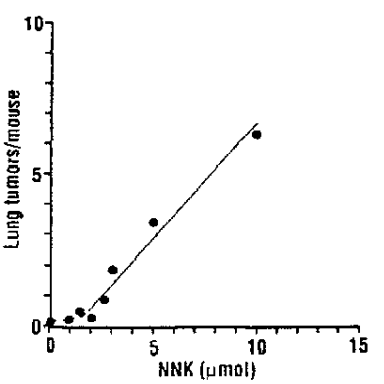

Fig. 5. Tumor multiplicity in groups of 20 A/J mice given various single i.p. doses of NNK in saline and sacrificed 16 weeks later. Data are from Table 4.

NNKOAc. Adduct levels were then compared to the tumorigenic activities of these treatments in order to see if there was a correlation between tumorigenic activity and O⁶-methylguanine formation and persistence.

Levels of O⁶-methylguanine following exposure to various doses of NNK are presented in Table 3. In general, there was a dose-dependent increase in the amount of O⁶-methylguanine measured at all time points. Substantial levels of O⁶-methylguanine were observed in all of the NNK dose groups 4 and 96 h after treatment. However, adduct levels in animals treated with 1, 1.5, or 2 μmol NNK fell below the detection limits at 14 days, despite similar adduct levels at 4 h in the 1.5–3-μmol groups.

The tumorigenic activities of these doses of NNK are summarized in Table 4. The lowest three doses were only slightly tumorigenic, if at all. The relationship between dose and tumor multiplicity is illustrated in Fig. 5. The dose-response curve appears to be biphasic with a break at approximately 2 μmol.

O⁶-Methylguanine concentrations detected at various time points after exposure to saline solutions of AMMN ± NNKOAc are listed in Table 5. A dose-dependent increase in adduct formation was observed. Inclusion of 4.2 μmol NNKOAc led to a significant increase of O⁶-methylguanine at all time points and with all doses of AMMN investigated.

One way in which NNKOAc could enhance lung DNA methylation by AMMN is by increasing the amount of AMMN that reaches the lung. This increase could be achieved through competition of NNKOAc and AMMN for esterase hydrolysis at the injection site and in blood. This hypothesis was tested by measuring lung alkylation by AMMN given with AcPB. AcPB is hydrolyzed by esterases to HPB (14); therefore, it should compete with AMMN for esterases. Neither AcPB nor its hydrolysis product, HPB, should interact with DNA. While both AcPB and HPB elevated levels of DNA methylation by AMMN at 4 h, they had no apparent effect on O⁶-methylguanine persistence (Table 6). Therefore, the ability of NNKOAc to increase methylation by AMMN is not due to an elevation

Table 5 *O⁶-Methylguanine levels in mouse lung DNA 4, 96, and 336 h after AMMN ± NNKOAc treatment[a]*

Compound (μmol)	pmol O⁶-methylguanine/μmol guanine[b]		
	4 h	96 h	336 h
AMMN (0.5)	3.3 ± 0.7	3.3[c]	2.1 ± 0.6
AMMN (0.5) + NNKOAc (4.2)	18.1 ± 5.4	9.7 ± 1.3	5.8 ± 2.0
AMMN (1.0)	15.5 ± 4.3	10.8 ± 4.4	5.5 ± 2.3
AMMN (1.0) + NNKOAc (4.2)	36.4 ± 8.0	26.0 ± 5.9	16.6 ± 3.3
AMMN (1.5)	11.9 ± 3.3	18.7 ± 8.3	11.7 ± 3.8[d]
AMMN (1.5) + NNKOAc (4.2)	37.8 ± 12.7	42.4 ± 9.5	23.6 ± 2.8[d]
AMMN (2.0)	34.7 ± 12.3	37.9 ± 14.8	13.2 ± 6.5

[a] Groups of mice were treated with the appropriate compounds at the indicated dose in saline (i.p.) Each group was divided into 3 subgroups for sacrifice at 4, 96, or 336 h after treatment. Lungs from 3 mice were pooled for DNA isolation. See "Materials and Methods" for details.
[b] Mean ± SD.
[c] Mean of two samples.
[d] Mean of four samples.

Table 6 *O⁶-Methylguanine levels in mouse lung DNA and corresponding tumor yields in mice treated with AMMN alone or in the presence of NNKOAc, AcPB, or HPB*

Compound[a]	O⁶-Methylguanine (pmol/μmol guanine)[b]			Lung tumors/mouse[c]
	4 h	96 h	336 h	
AMMN	4.4 ± 0.9	<2.2[d]	<2.2[d]	1.2 ± 0.4
AMMN + NNKOAc	18.1 ± 5.4[e]	9.7 ± 1.3[e]	5.8 ± 2.0[e]	2.6 ± 0.4[e]
AMMN + AcPB	14.3 ± 4.7[e]	—	<2.5[d]	0.6 ± 0.2
AMMN + HPB	9.2 ± 2.2[e]	—	<2.6[d]	0.7 ± 0.2

[a] Groups of 9 mice received a single i.p. dose of 0.5 μmol AMMN ± 4.2 μmol NNKOAc, AcPB, or HPB (in saline) and were sacrificed at the indicated times. Lungs from three animals were pooled for DNA isolation. See "Materials and Methods" for details.
[b] Three samples ± SD.
[c] Groups of 20 mice (20.1 ± 1.6 g) were sacrificed at 16 weeks following a single i.p. dose of the indicated compound(s) and lung adenomas were counted. The tumor counts were expressed as the mean ± SE.
[d] Detection limits were 0.45 pmol O⁶-methylguanine. The amount of DNA analyzed ranged from 0.2 to 0.5 mg.
[e] Statistically different from AMMN alone, Student's t test, $P < 0.05$.
— Not determined.

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Table 7 *Dose response for lung adenomas in A/J mice treated with AMMN ± NNKOAc*[a]

AMMN (μmol)	−4.2 μmol NNKOAc			+4.2 μmol NNKOAc		
	% of mice with tumors	Tumors/mouse	Tumors/O^6-methylguanine at 96 h[b]	% of mice with tumors	Tumors/mouse	Tumors/O^6-methylguanine at 96 h[b]
0.0	10	0.1 ± 0.1	—[c]	35	0.5 ± 0.2	—
0.5	60	1.2 ± 0.4	0.36	85	2.6 ± 0.4[d]	0.26
0.75	70	1.7 ± 0.6	—	85	4.3 ± 0.8[d]	—
1.0	80	3.5 ± 1.6	0.32	100	7.5 ± 0.8[d]	0.29
1.25	95	6.6 ± 1.6	—	100	11.5 ± 1.1[d]	—
1.5	95	8.9 ± 1.7	0.48	100	13.5 ± 1.1[d]	0.32
2.0	100	14.6 ± 1.9	0.39	95	18.3 ± 1.8	—

[a] Groups of 20 mice (average starting weight, 20.1 ± 1.6 g) were sacrificed at 16 weeks following a single i.p. dose of the indicated compound(s) and lung adenomas were counted. The tumor counts were expressed as the mean ± SE.
[b] The ratios were obtained using O^6-methylguanine levels presented in Table 5.
[c] —, not determined.
[d] Statistically different from AMMN alone. Student's *t* test. $P < 0.05$.

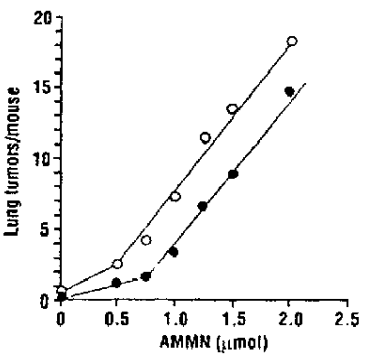

Fig. 6. Dose response for lung tumor formation in A/J mice treated with AMMN ± 4.2 μmol NNKOAc. ●, mean value for groups receiving only AMMN; ○, mean value of the groups receiving both AMMN and NNKOAc. Data are from Table 7.

Table 8 *Relationship between tumors/mouse and O^6-methylguanine levels in mouse lungs 96 h following exposure to NNK or AMMN ± NNKOAc*

	Tumors/O^6-methylguanine at 96 h[a]
NNK	0.34 ± 0.04[b] (3)
AMMN	0.40 ± 0.08[c] (4)
AMMN + NNKOAc	0.29 ± 0.03[c] (3)
Overall	0.34 ± 0.07

[a] Mean ± SD. Numbers in parentheses, number of values.
[b] From Table 4 using values from groups treated with greater than 2.5 μmol NNK.
[c] From Table 7.

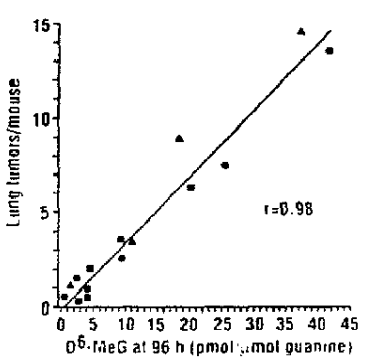

Fig. 7. Relationship between tumor formation and the concentration of O^6-methylguanine (O^6-MeG) in lung DNA 96 h after treatment with NNK (■), AMMN (▲), or AMMN + 4.2 μmol NNKOAc (●). NNK [illegible] from Table 5 and AMMN ± NNKOAc data are from Table 8.

of the effective AMMN concentration or to effects caused by the NNKOAc metabolite, HPB.

A comparison of adduct levels generated from AMMN ± NNKOAc to those produced from 10 μmol NNK demonstrates that different doses of AMMN ± NNKOAc mimic [illegible]K-derived O^6-methylguanine levels depending on the time point used (Tables 3 and 5). The combination of 0.5 μmol AMMN + 4.2 μmol NNKOAc most closely reproduces O^6-methylguanine levels generated by 10 μmol NNK at 4 h whereas 1.5 μmol AMMN or 1.0 μmol AMMN + 4.2 μmol NNKOAc are better models at 96 h. AMMN (1.5 μmol) also yields levels comparable to those produced by 10 μmol NNK 2 weeks after treatment.

The results of the bioassay for tumorigenicity of AMMN ± NNKOAc are summarized in Table 7. There was a biphasic dose response in tumor formation by AMMN (Fig. 6), as observed for NNK (Fig. 5). Addition of 4.2 μmol NNKOAc significantly enhanced the tumorigenicity of AMMN. This increase was not observed when AcPB or HPB was administered with 0.5 μmol AMMN (Table 6). The slopes of the dose-response curves for tumor formation by AMMN, AMMN + NNKOAc, and NNK were similar.

When the tumorigenic activities of various doses of AMMN ± NNKOAc were compared to that of 10 μmol NNK, 1.25 μmol AMMN or 1.0 μmol AMMN + 4.2 μmol NNKOAc most closely approximated the tumor yield of 10 μmol NNK. These doses are within the range of doses found to produce similar O^6-methylguanine levels at 96 h and 2 weeks as observed with 10 μmol NNK (1.5 μmol AMMN or 1.0 μmol AMMN + 4.2 μmol NNKOAc). The most consistent relationship between O^6-methylguanine levels and tumorigenic activities of various doses of AMMN ± NNKOAc was observed using adduct levels at 96 h. The ratio of lung tumors/mouse to levels of pulmonary O^6-methylguanine at 96 h was similar for all AMMN ± NNKOAc dose groups as well as for NNK doses greater than 2.5 μmol (Table 8). The strong correlation between tumors/mouse and adduct levels at 96 h ($r = 0.98$; Fig. 7) demonstrates that NNK activity can be reproduced by AMMN ± NNKOAc at doses that generate a comparable O^6-methylguanine concentration at this time point.

DISCUSSION

The relative importance of DNA pyridyloxobutylation and methylation pathways in NNK-induced lung tumorigenesis was investigated in the A/J mouse using model compounds that only pyridyloxobutylated (NNN and NNKOAc) or methylated (AMMN) DNA. The tumorigenicities of these compounds were assessed at various doses, including those that pyridyloxobu-

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tylated or methylated DNA to an extent similar to that of 10 μmol NNK. Under these conditions, NNN was inactive and NNKOAc was only weakly tumorigenic. AMMN was very active in inducing lung tumors in A/J mice. These results are consistent with the observation that deuterium substitution on the methylene carbon of NNK blocks DNA methylation as well as tumor formation in A/J mouse lung (17). Methyl deuteration did not lower NNK activity. These studies indicate that DNA methylation is a critical event in the initiation of A/J mouse lung tumors by NNK.

Persistence of O^6-methylguanine appears to be important for tumorigenic activity since the inflection in the NNK tumorigenicity dose-response study occurs at the same dose at which we begin to detect measurable levels of O^6-methylguanine at 14 days following NNK exposure. The enhanced persistence at higher doses may result from saturation of the repair enzyme, O^6-methylguanine-DNA transmethylase. Previous reports indicated that Clara cells accumulate O^6-methylguanine in rat lung following NNK treatment (10 mg/kg/day) and that NNK treatment effectively eliminates any measurable transferase activity in these cells (18). Consequently disappearance of O^6-methylguanine is much slower in Clara cells than in other pulmonary cell types (18). A strong correlation was found between the O^6-methylguanine levels in Clara cells and lung tumor incidence in rats (9).

While our experiments in mice examine levels of DNA alkylation in whole lung and do not account for cellular selectivity in NNK activation, we also observed a strong correlation between tumors/mouse and O^6-methylguanine levels measured at 96 h for AMMN ± 4.2 μmol NNKOAc, as well as for NNK (r = 0.98; Fig. 7). Therefore, NNK lung tumorigenic activity can be reproduced when mice were treated with a dose of AMMN ± NNKOAc that produced comparable O^6-methylguanine levels in lung DNA 96 h after exposure.

Activation of K-*ras* oncogene appears to be important in both chemically induced and spontaneous lung tumors in A/J mice (19). While the mutational spectrum of activated K-*ras* oncogene in spontaneously generated tumors was nonspecific, mutations in this gene from A/J mouse lung tumors induced by NNK predominantly involved G-C to A-T transitions in the second base of codon 12 (19). This mutational event is consistent with formation of O^6-methylguanine (20, 21). Therefore, the strong correlation between the persistence of O^6-methylguanine and tumor formation is likely connected to the importance of K-*ras* oncogene activation in A/J mouse lungs.

The weak activity of pyridyloxobutylating agents in this animal model suggests that pyridyloxobutyl DNA adducts are not activators of the K-*ras* oncogene. AMMN and NNKOAc have similar spectra of mutagenic activity in various *Salmonella* strains[5]; *i.e.*, both AMMN and NNKOAc were active in TA100 and TA98 but not TA102.[5] However, the chemical nature of the pyridyloxobutyl adducts as well as the types of mutations generated by these adducts are unknown. Pyridyloxobutyl adducts may generate mutation types different from methyl adducts since the mutational spectrum of NNK in *Escherichia coli lacI* gene was considerably more complex than that of AMMN (22). The apparent inability of NNKOAc to activate K-*ras* oncogene does not preclude its ability to activate other oncogenes.

The observation that DNA methylation is the critical event in tumor induction by NNK in A/J mouse lung does not mean that this will be the case in other NNK-induced tumors. The A/J mouse is predisposed to form lung tumors and K-*ras* oncogene activation appears to be critical for lung tumorigenicity in this species.

There are differences in oncogene activation in tumors generated by NNK in different animal species and tissues. For example, K-*ras* activation is important in NNK-induced lung tumors in A/J and C3H mice (8, 19), but does not appear to be important in liver tumor induction by NNK in C3H mice (8) or in lung tumor induction in F344 rats following chronic NNK exposure (23). Furthermore, the ability of NNN to induce esophageal and nasal tumors (24, 25) suggests that the pyridyloxobutylation pathway is capable of inducing tumors, possibly through activation of oncogenes other than K-*ras* or by other mechanisms. Additional studies are required to determine the role of pyridyloxobutylation in other animal models.

The pyridyloxobutylation pathway appears to play an important role in increasing the activity of the methylation route in A/J mouse lung since NNKOAc was found to markedly increase the tumorigenicity of AMMN. This enhancement was attributed to its ability to increase AMMN-derived O^6-methylguanine levels and their persistence. The mechanism by which these levels are increased is unknown. NNKOAc does not appear to elicit its effects by merely increasing the effective concentration of AMMN through competition for esterases. AcPB, which would compete with NNKOAc for esterase hydrolysis, did not affect persistence of O^6-methylguanine generated by AMMN. In addition, HPB was not able to significantly affect persistence of AMMN-derived O^6-methylguanine. This observation indicates that HPB, the principle metabolite of NNKOAc, was not involved in the enhanced methyl adduct levels seen when NNKOAc was coadministered with AMMN. Consistent with this observation, neither AcPB nor HPB were able to enhance the tumorigenic activity of 0.5 μmol AMMN. Therefore, it is likely that NNKOAc enhances methylation levels and consequently the tumorigenic activity of AMMN, through either protein or DNA pyridyloxobutylation. One possibility is that pyridyloxobutylation of the repair enzyme O^6-methylguanine-DNA methyltransferase occurs, thus impairing its ability to reverse methylation damage at the O^6-position of guanine.

The ability of NNKOAc to increase the alkylation efficiency of AMMN suggests a possible role for the pyridyloxobutylation pathway in NNK tumorigenesis. Since pyridyloxobutylation by NNKOAc increases the methylation efficiency of AMMN, it is possible that NNK pyridyloxobutylation can increase the efficiency of NNK methylation. Belinsky *et al.* (26) reported that the methylation efficiency of NNK was markedly increased at lower doses of NNK. This effect was not observed with DMN. This observation was explained by differences in isozyme activation of NNK and DMN. Another contributing factor may be that methylation by NNK at lower doses is enhanced by the pyridyloxobutylation pathway. Since DMN lacks this route, the enhancement would not be observed.

In summary, our results demonstrate that methylation of lung DNA is a crucial step in A/J mouse lung tumorigenesis by NNK. Persistence of O^6-methylguanine is required for tumor formation. These studies also indicate that DNA pyridyloxobutylation by NNK may be capable of prolonging the lifetime of this promutagenic base in lung DNA.

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